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Pediatric Antibody Response to Community-Acquired *Staphylococcus aureus* Infection Is Directed to Panton-Valentine Leukocidin▽

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We examined the antibody responses of pediatric patients infected with community-associated *Staphylococcus aureus* isolates. The data show that patients infected with Panton-Valentine leukocidin (PVL)-positive strains developed a dominant immunoglobulin G anti-PVL antibody response that correlates with markers of inflammation.

Staphylococcus aureus is one of the most common bacteria causing skin and soft tissue infections as well as invasive infections, e.g., osteomyelitis, septic arthritis, infective endocarditis, and complicated pneumonia, in humans (14). Over the past decade, the USA300 *S. aureus* pulsed-field type has become the dominant *S. aureus* lineage causing community-associated methicillin-resistant *S. aureus* (CA-MRSA) infections worldwide (3–8, 13, 15, 16, 20); this lineage encodes the Panton-Valentine leukocidin (PVL), a cytotoxin that has been associated with severe *S. aureus* infections in humans (3–8, 13, 15, 16, 20). We examined the anti-PVL antibody responses in patients diagnosed with *S. aureus* infections (Table 1) and compared these responses to those against other USA300 virulence factors.

Sera for antibody analysis were collected at the time of admission from pediatric patients with either skin and soft tissue infections (SSTI), invasive bone infections (osteomyelitis), or pneumonia. Sera from healthy 4- to 6-year-old children (collected in 1991) served as negative controls.

The serum antibody responses to the following *S. aureus* recombinant factors were measured: extracellular fibrinogen-binding protein from amino acid positions 35 to 165 ($Efb_{35\text{-}165}$) (10, 11), MHCII analog protein from amino acid positions 50 to 237 ($Map19_{50\text{-}237}$) (12), LukF-$PV_{25\text{-}325}$ and LukS-$PV_{29\text{-}312}$ (9), clumping factor B from amino acid positions 201 to 542 ($ClfB_{201\text{-}542}$) (19), collagen-binding adhesin 35 from amino acid positions 29 to 334 ($CNA35_{29\text{-}334}$) (22), or the pGEX-2T (GE Life Sciences, Piscataway, NJ) vector for fibronectin-binding protein A from amino acid positions 620 to 881 ($FnbpA_{620\text{-}881}$) (17). In addition, $LukD_{27\text{-}327}$, $LukE_{40\text{-}311}$, gamma hemolysin A from amino acid positions 30 to 309 ($HlgA_{30\text{-}309}$), $HlgB_{27\text{-}325}$, and $HlgC_{30\text{-}315}$ were cloned and expressed for this study. Alpha toxin was purchased from List Biological Laboratories (Campbell, CA), and the LukS-PV signal peptide (formyl-Met-Val-Lys-Lys-Arg-Leu-Leu-Ala-Ala-Thr-Leu-Ser-Leu-Gly-Ile-Ile-Thr-Pro-Ile-Ala-Thr-Ser-Phe-His-Glu-Ser-Lys-Ala-OH) and α3-phenol soluble modulin (α3-PSM; formyl-Met-Glu-Phe-Val-Ala-Lys-Leu-Phe-Lys-Phe-Phe-Lys-Asp-Leu-Leu-Gly-Lys-Phe-Leu-Gly-Asn-Asn-OH) were synthesized by AnaSpec, Inc. (San José, CA) (21). These factors were chosen as representative members of the adhesin, toxin, and immunomodulator families. The collagen adhesin CNA (not encoded in the USA300 genome) was selected as a negative control.

Specific antibody responses were characterized by using alkaline phosphatase-conjugated (1:5,000) goat anti-human whole immunoglobulin G (IgG) antibodies; IgA antibodies; or

TABLE 1. Demographic characteristics of children with *S. aureus* infections

Characteristic	Result for children with:	
	Invasive infections[a]	SSTI[b]
Mean age ± SE (yr)	6.58 ± 1.03	7.74 ± 0.92
No. (%) of isolates		
pvl positive	21 (64)	24 (69)
pvl negative	9 (33)	2 (6)
ND[c]	3 (9)	9 (25)
MRSA	13 (40)	20 (57)
MSSA	20 (60)	6 (17)
ND	0 (0)	9 (26)
Previous infection		
Yes	10 (30)	19 (54)
No	21 (64)	7 (20)
Unknown	2 (6)	9 (26)
Race		
Caucasian	15 (46)	15 (43)
Black	7 (21)	11 (31)
Hispanic	6 (18)	8 (23)
Other[d]	5 (15)	1 (3)

[a] Invasive infections were defined as infections of the bone ($n = 28$), myositis ($n = 3$), and pneumonia ($n = 2$).
[b] SSTIs were defined as abscesses ($n = 26$), cellulitis ($n = 2$), and lymphadenitis ($n = 4$).
[c] ND, not determined.
[d] Other, three Asian and two unknown.

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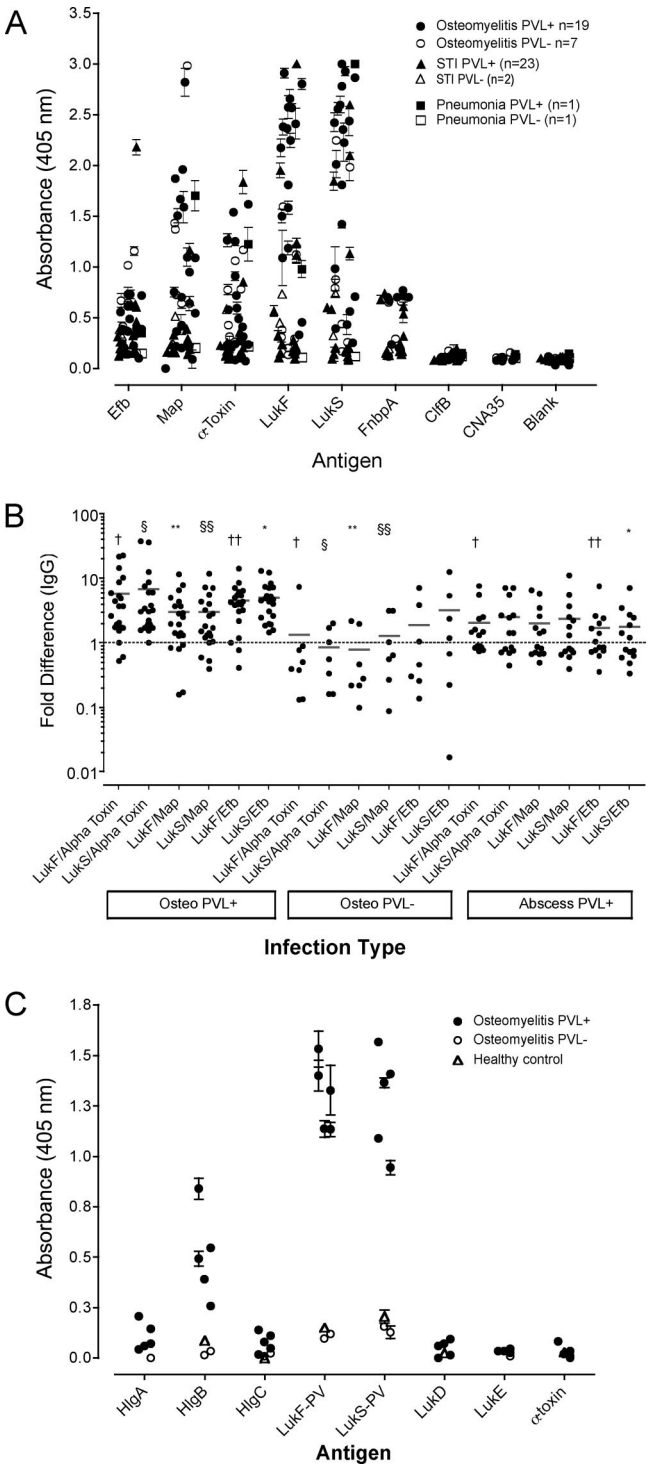

FIG. 1. Antibody reactivities to *S. aureus* virulence proteins. ELISA plate wells coated with *S. aureus* proteins were incubated with a 1:1,000 dilution of patient serum and were then probed with a 1:5,000 dilution of alkaline phosphatase-conjugated anti-IgG antibody. The plates were developed for 30 min in the dark and then read at 405 nm. The data are expressed as the means ± standard errors for triplicate wells per patient. (A) IgG responses to Efb, Map, alpha toxin, LukF-PV, LukS-PV, ClfB, CNA35, and FnbpA in patients with different disease presentations. (B) Log difference in anti-PVL antibody titers. The data are expressed as the log difference between the mean OD readings obtained for individual patients described above. Statistical differences were determined by the unpaired *t* test with Welch's correction; and differences between groups are indicated by the corresponding symbols: †, $P \leq 0.02$; §, $P \leq 0.02$; **, $P \leq 0.004$; §§, $P \leq 0.04$; ††, $P \leq 0.001$; *, $P \leq 0.001$. (C) Antibody reactivities to *S. aureus* toxins. ELISA plate wells were coated with 1 μg/well of either HlgA, HlgB, HlgC, LukF-PV, LukS-PV, LukD, LukE, or alpha toxin. The data are expressed as the means ± standard deviations for triplicate wells per patient. PVL+, PVL positive; PVL−, PVL negative; STI, soft tissue infection; Osteo, osteomyelitis.

mouse isotype-specific anti-human IgG1, IgG2, IgG3, IgG4, or IgM antibodies (Zymed Laboratories, San Francisco, CA). Immulon-1B microtiter plates (Dynatech Laboratories, Chantilly, VA) were coated overnight at 4°C with 1 μg of each recombinant *S. aureus* antigen, and enzyme-linked immunosorbent assays (ELISAs) were carried out as described previously by using a 1:1,000 dilution of patient serum (2).

The levels of IgG antibodies to LukS-PV and LukF-PV were significantly higher ($P < 0.01$) in children with invasive infections (median optical density [OD], 0.98; OD range, 0.12 to 3.00) than in children with SSTI (median OD, 0.47; OD range, 0.09 to 2.60) (Fig. 1A). A similar trend ($P = 0.06$) for the levels of IgG antibodies to alpha toxin was noted in the two groups, but the levels of antibodies to alpha toxin were lower than those to LukS-PV in each group (Fig. 1A). No reactivity to the LukS-PV signal peptide or α3-PSM was detected. In patients with invasive infections, the levels of IgG antibodies to LukS-PV correlated with the number of days prior to admission that the patient was ill ($r = 0.44$, $P = 0.023$) and the C-reactive protein (CRP) values at the time of admission ($r = 0.42$, $P = 0.03$).

There was a trend for the anti-LukS-PV IgG antibody levels to correlate with the erythrocyte sedimentation rate (ESR) at admission ($r = 0.35$, $P = 0.08$). In contrast, the levels of antibodies to alpha toxin did not correlate with the duration of illness prior to admission or the CRP value at admission, but a trend for a correlation with the ESRs at admission was noted ($r = 0.39$, $P = 0.09$). There was no correlation between the anti-LukS-PV or anti-alpha toxin IgG antibody response and the patient's age or between the anti-LukS-PV and anti-alpha toxin IgG antibody responses.

The anti-Map IgG antibody levels in patients with invasive infections (median OD, 0.64; OD range, 0.09 to 2.98) were also significantly greater ($P = 0.002$) than those in patients with SSTI (median OD, 0.25; OD range, 0.13 to 1.91). The anti-Efb IgG antibody levels were lower than the levels of the other IgG antibodies and were not different between the patient groups. The levels of IgA antibodies to the protein panel were not significantly different between groups, and IgA antibodies were present at lower levels than the IgG antibodies (data not shown). Antibody responses to CNA and ClfB were not detected in the patients (Fig. 1A).

As described above, patients from all groups had various responses to most of the virulence factors tested. Patients with *pvl*-positive osteomyelitis had anti-LukF-PV and anti-LukS-PV IgG antibody responses that were more dominant than those of patients diagnosed with *pvl*-negative osteomyelitis or *pvl*-positive SSTI. This was examined further by comparing the anti-LukF-PV and anti-LukS-PV IgG antibody responses of each patient to their respective IgG responses to alpha toxin,

Map, and Efb (factors that consistently elicited measurable responses). Patients with *pvl*-positive osteomyelitis on average had statistically higher IgG anti-LukF-PV and anti-LukS-PV responses (approximately fivefold greater than their responses to alpha toxin, Map, or Efb) (Fig. 1B). In contrast, patients infected with *pvl*-negative isolates had alpha-toxin, Map, and Efb responses higher than their respective anti-LukF-PV and anti-LukS-PV responses (Fig. 1B). These data suggest that patients infected with PVL-positive isolates develop a dominant anti-PVL response at the expense of other factors.

The IgG isotype (i.e., IgG1, IgG2, IgG3, and IgG4) reactivities against the virulence proteins described above were determined to define the differences in Ig responses between groups; however, no significant differences were observed (data not shown).

Since the dominant antibody responses observed were toward LukF-PV and LukS-PV, we examined the possibility that the magnitude of this response was due to the presence of cross-reactive antibodies generated against structurally similar factors, e.g., other pore-forming toxins, by testing patient sera from high and low anti-PVL IgG antibody responders for reactivity to either LukD, LukE, alpha toxin, HlgA, HlgB, or HlgC. Sera from five patients with *S. aureus* (*pvl*-positive) osteomyelitis responded exclusively to LukF and LukS and had some reactivity to HlgB (Fig. 1C). Serum obtained from patients with infections caused by *pvl*-negative isolates or from controls were nonresponsive to the toxins tested (Fig. 1C), indicating that the anti-LukF and anti-LukS responses were primarily due to a specific humoral response to these antigens.

Although the protein panel tested for Ig reactivity represents a small fraction of the total target antigens with the potential of eliciting a humoral response against *S. aureus*, the antigens selected were chosen because they are virulence factors in humans and in animal models of disease (1, 9, 12, 14, 18). This report is the first describing the antibody responses to selected *S. aureus* antigens in pediatric patients with either SSTI or invasive infections caused by CA-MRSA isolates of the USA300 lineage. Patients with invasive infections developed more dominant and specific anti-LukF-PV and anti-LukS-PV responses than patients with SSTI. The titers of the antibodies to these determinants correlated with markers of inflammation, the significance of which remains to be understood.

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ADDENDUM IN PROOF

After this paper was accepted for publication, we cloned and expressed a new LukE construct (amino acids 29 to 311) that we tested for antibody reactivity as described for Fig. 1C. Sera collected from patients infected with PVL-negative isolates did not react significantly to this construct, $LukE_{29\text{-}311}$. However, serum from patients infected with PVL-positive isolates bound to $LukE_{29\text{-}311}$ at levels similar to those observed for LukS. These observations do not change the conclusions derived from the data presented. Rather since only patients infected with PVL-positive isolates responded to LukE, whereas patients infected with PVL-negative isolates (which also express LukE but not either LukE nor LukS) did not have antibodies reactive to either LukF or LukS, the results obtained with this expanded construct suggest that antibodies raised against LukS cross-reacted with LukE, not vice versa.

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